STUDIES ON SHELL FORMATION. I. MEASUREMENT OF THE RATE OF SHELL FORMATION USING Ca^{45} [1, 2]

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Shell growth is commonly measured in ecological and conservation studies as a linear increase occurring in one or a few weeks, or during a shorter period if growth is very rapid. For such studies measurements of growth increments of the order of one millimeter are usually sufficiently sensitive. For the experimental analysis of shell formation, the availability of a method of a different order of sensitivity would be distinctly advantageous and at the same time would make possible new approaches to the study of shell growth. The extreme sensitivity of radioactivity measurements suggested the use of radioisotopes. The determination of the rate at which Ca^{45} is incorporated into growing shell should give a measure of the amount of mineral substance deposited during shorter intervals than would be otherwise possible. The isotope method would also permit for the first time the quantitative measurement of mineral deposition in localized areas of the inner shell surface.

In the present series of studies the radioisotope method has been utilized in the analysis of several phases of shell formation. These include: (1) the quantitative measurement of calcium deposition; (2) rate of transfer of calcium through the mantle; (3) the distribution of inorganic and organic material deposited on the inner shell surface as studied by autoradiographic and histochemical methods; and (4) the exchange of calcium between shell and sea water in the absence of living tissues. This study deals with the first of these problems and is specifically concerned with the examination of the suitability of the isotope method for measuring calcium deposition in the living oyster under standardized conditions. These experiments demonstrate that the isotope method can be used to measure the deposition of calcium and that growth increments occurring in four hours are measureable.

METHODS

Oysters (*Crassostrea virginica* Gmelin[3]) were collected from Bogue Sound near Beaufort, N. C., and kept below low tide level at the laboratory until ready for use. Those to be used for a particular experiment were matched approximately as to size (ca. 8–9 cm. in length) and the surfaces scrubbed free of foreign matter. After drying briefly they were coated with fingernail polish to prevent uptake of Ca^{45} by

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[3] Previously *Ostrea virginica*. Re-named by Gunter (1950).

the outer surface of the shell.[4] The coated oysters were kept below low tide level for several hours or overnight to remove all traces of the solvent.

Single oysters were placed in one liter of sea water in large flat glass dishes and shell movements were recorded on a kymograph throughout the experimental period. Such records provided data on periods of closure as well as indicating normalcy of shell movements. If shell movements were normal, the sea water was renewed and Ca^{45} added to give an activity of 6×10^5 counts per minute per liter of sea water. The Ca^{45} was of high specific activity or carrier-free so that the calcium content of the sea water was not increased appreciably. The radioactive sea water was renewed every four hours. The temperature range was 21.2 to 25.4° C. for the entire period of study though approximately constant for each experiment. Sea water salinity was approximately 35 parts per thousand.

After intervals of 8, 16 and 24 hours, oysters were removed from the sea water containing Ca^{45} and the shells prepared for determinations of the radioactivity of the inner shell surface. The mantle was removed and the region of muscle attachment carefully scraped free of material. The shell was then rinsed thoroughly with a fine stream of distilled water from a wash bottle. Previous experiments had shown that this treatment was sufficient to remove all adhering material which might be radioactive and was as effective as scrubbing with a stiff brush. The radioactivity was measured for circular areas of 6.2 $cm.^2$ in the following regions: extreme posterior end; center which included a portion of the site of muscle attachment; and the anterior end immediately posterior to the hinge. Essentially constant geometry was maintained by means of a special holder in which the shell was held close to the window of the counting tube for the measurements just mentioned or against a brass plate with a circular aperture of 1 cm^2. for more exact localization of radioactivity. Because of geometrical difficulties with the cupped left valve, measurements were confined to the less curved right valve. A thin window counting tube (1.9 mg./cm^2.) was used.

Carefully cleaned right shells were included in all experimental solutions in order to obtain an estimate of the radioactivity of the shell resulting from exchange between sea water and shell as contrasted with deposition by the living oyster. The values for the living oyster were corrected for exchange in each instance.

RESULTS

Figure 1 shows the Ca^{45} deposited after various intervals in the posterior region of shell of 60 oysters. The mean Ca^{45} deposition was directly proportional to the period of exposure to the isotope. Marked individual variation is apparent and will be discussed later. Graphs of the center and anterior regions gave a similar picture but differed quantitatively from the posterior region. Calcium deposition could be measured after four hours in radioactive sea water under these conditions, as suggested in Figure 1 and as demonstrated in experiments not reported here. With higher concentrations of Ca^{45}, deposition would be measurable after still shorter intervals.

The deposition rate was not uniform over the shell surface; the relative mean depositions after 24 hours for the three regions measured were: posterior 1.0, center 0.55 and anterior 0.58. The regional differences were investigated further by making measurements of Ca^{45} activity on several circular areas of 1 cm.^{2} (Fig. 2). The positions of the columns indicate the regions for which radioactivity was determined and the height gives the relative deposition. The non-uniformity of rate of deposition measured in small areas is more marked than with the larger areas mentioned above. This general pattern, which represents the means from 8 oysters, also characterized the individual shells. The main features of the regional differences are the following: (1) The differences in deposition in adjacent small areas may be great. And the pattern of rates obtained will accordingly depend upon the size of the area included in the individual measurements, as may be seen by comparing the values for the three larger areas with those in Figure 2. (2) The rate of deposition in the posterior portion was relatively high, although the thin posterior edge was low. (3) The portions near the periphery of the shell exhibited a higher rate than the interior. An autoradiographic study, which will more exactly define the pattern of deposition, is now in progress.

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[4] P^{32} is taken up by nail polish though not by a coating of wax.

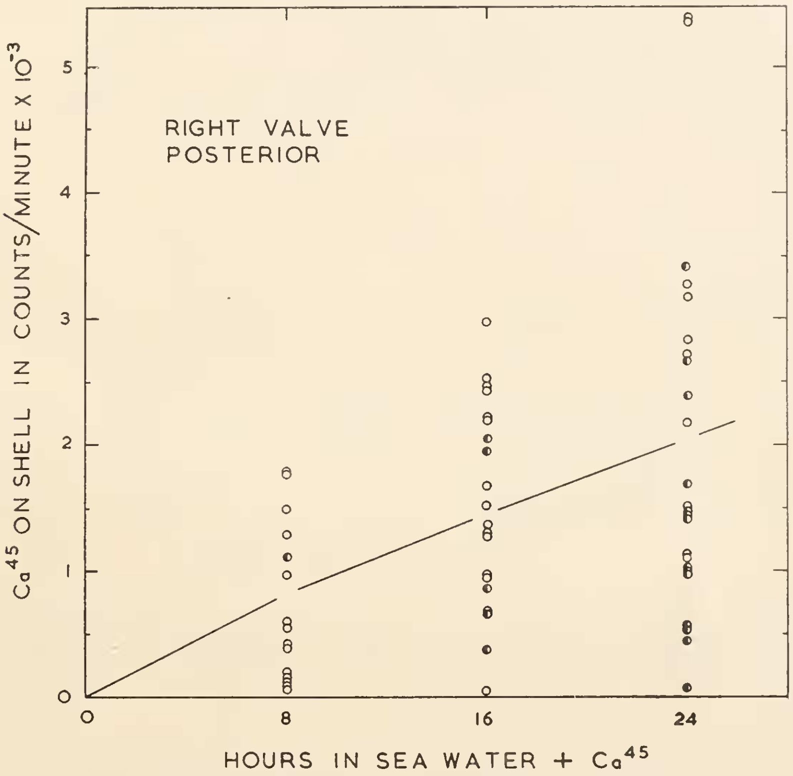

FIGURE 1. Ca^{45} deposition by the living oyster. Each point represents the radioactivity of a circular area of 6.2 cm.^{2} at the extreme posterior end on the inner surface of the right valve of one oyster. The line connects the mean values for Ca^{45} deposition for each interval. The half closed circles represent Ca^{45} deposited by oysters which remained closed for more than 25% of the experimental period.

An approximation of the weight of calcium deposited can be made by comparing the radioactivity of the sea water and the shell. Such an estimate becomes possible when the following conditions are operative:

1. *The measured radioactivity of the shell is directly proportional to the total* Ca^{45} *deposited in a given interval.* This will be the case provided the layer of new material is sufficiently thin that the radiation from the calcium first laid down is not absorbed by the salts and conchiolin deposited later. The estimated amounts of $CaCO_3$ deposited (see method below) in the intervals used are such that the factor of self-absorption can probably be neglected (Comar *et al.*, 1951) except with the higher values found with 24 hours exposure [5] (Fig. 1).

2. *The calcium being deposited is of the same specific activity as the sea water.* Other studies (Jodrey, 1952) have shown that the Ca^{45} in the mantle has reached equilibrium in about one hour. The fact that the mean radioactivity is a straight line function of time (Fig. 1) also indicates that equilibrium is attained relatively quickly.

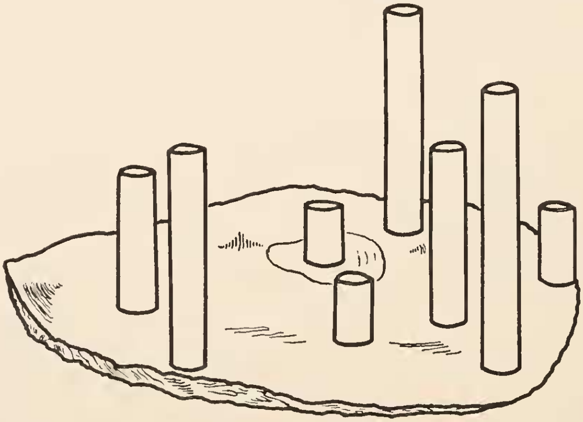

FIGURE 2. Distribution of newly deposited calcium on the inner surface of shell. Ca^{45} deposited after 24 hours was measured on several one cm.^{2} regions of the shell as indicated by the position of the columns. The height of the columns shows the relative amounts of Ca^{45} deposited. The site of the muscle attachment may be taken as 1. The figures represent average values for 8 shells.

3. *The difference in backscatter in measurements of radioactivity of sea water and shell is known.* Radioactivity measurements of the sea water were made on copper planchets whereas measurements of shell were made against the $CaCO_3$ of the shell itself. The higher atomic number of copper as compared with calcium would give an increased backscatter and an underestimation of the calcium deposition of the order of 8 or 9% (Glendennin and Solomon, 1950).

4. *The specific activity of the sea water is not appreciably changed by unlabeled calcium from the tissues and body fluids.* Error from this cause would be negligible

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[5] With the mean deposition rate of 9.0×10^{-3} mg. $CaCO_3/cm.^2/hr.$ found in the posterior region, the limiting value of approximately 0.5 mg./cm.^{2} would be reached in about 56 hours. For the shells showing most active deposition (Fig. 1) the limiting value has already been exceeded slightly in 24 hours.

because of different orders of magnitude (ca. 400 mg. Ca per liter of sea water and ca. 7 mg. Ca in the oyster (Sherman, 1947)). The renewal of the radioactive sea water every four hours would further minimize this factor.

Under the conditions defined above the calcium deposition can be calculated from the following relation:

$$D = \frac{A_s}{A_w} \times C,$$

where D is mg. Ca deposited cm.^{2}, A_s is counts/min./cm.^{2} on shell, A_w is counts/min./liter sea water, and C is mg. Ca/liter sea water.

Using this relationship without further correction, the mean amount of calcium deposited was calculated to be 9.0×10^{-3} mg./cm.^{2}/hr. for the posterior region of the right valve and 5.2×10^{-3} mg. for a similar area in the anterior region. If we assume for purposes of a rough estimate an average value of 7×10^{-3} mg. Ca/cm.^{2}/hr. for both valves, an oyster of 8.5 cm. in length with an internal shell surface of 70 cm.^{2} would deposit 0.92 gm. of $CaCO_3$ in a month under these particular conditions. If the $CaCO_3$ constituted about 90% of the shell weight, the total weight gain would be approximately one gram. Because of difference in species and unknown differences in conditions, a comparison with the results of others (Havinga, 1928; Masumoto *et al.*, 1934; Galtsoff, 1934) on shell growth in natural waters is scarcely possible.

The rate of deposition differed widely in different individuals (Fig. 1) as previously mentioned, even though oysters were of approximately the same size collected from the same site, treated identically, and exposed to radioactive sea water simultaneously. However, since a size range, although small, did exist, the calcium deposition for each period of exposure has been examined with respect to various parameters of shell size by plotting the amount deposited against length, width, area, weight, and average thickness (area/weight). The scatter of points in each case indicated the absence of significant correlation. It should be pointed out that inasmuch as individuals used were selected for similarity of size, the present findings in no way indicate an absence of correlation between rate of deposition and size for a greater size range. Orton (1935) has shown a negative correlation between relative growth rate and size in the English oyster, while a low degree of correlation between rate of growth and size and weight of oysters has been reported by Havinga (1928).

The erosion of the inner surface which takes place when mollusks remain closed for extended periods suggested the possibility of removal of previously deposited calcium during the relatively long periods of closure which occurred in some individuals. Also, if deposition continued during closure, it might proceed at a different rate. To investigate the effect of closure, the time that each of the 60 oysters remained closed was determined from the kymograph records of shell movements. The deposition in individuals which remained closed for more than 25% of the experimental period appeared to be about the same as those which remained open for a longer time except for the 24-hour period where less deposition took place in the posterior region. (See Fig. 1.)

The observed variability in growth rate is not peculiar to the conditions of these experiments, for great variability is also the common finding in oysters growing in natural waters (Havinga, 1928; Loosanoff and Nomejko, 1949; Butler, 1952).

DISCUSSION

The results presented indicate the feasibility of the use of Ca^{45} in studies of shell growth in the oyster. The use of radioisotopes offers two distinct advantages over conventional methods: (1) Extremely small growth increments such as those occurring in a few hours can be measured. (2) Differences in rate of deposition in localized areas can be determined. For comparative measurements of shell growth the radioactivity of a single area such as the posterior region can be used as an index of growth. Because of localized differences in deposition over the surface, total growth can be determined only by measurement of the radioactivity of the whole surface.

The isotope method for studying shell growth also imposes definite limitations. The experimental periods must be short in the case of rapid growth; otherwise absorption by layers overlying the material deposited previously will interfere with the measurement of the soft β radiation of the Ca^{45}. . Because of the expense of isotopes and for practical considerations, the volumes of the radioactive solutions will normally be small, thus placing restrictions on the type of studies which can be carried out conveniently. Moreover, particular consideration must be given to the maintenance of a favorable environmental situation with small volumes. As judged by normal shell movements this requirement appeared to be reasonably well fulfilled in the present studies by placing a single oyster in one liter of sea water in a shallow dish and changing the sea water every four hours. Radiation injury is probably not a complicating factor inasmuch as the radioactivity of the medium can be kept relatively low. An obvious limitation of the method is that successive measurements of growth cannot be carried out on a single individual.

In addition to the active deposition of Ca^{45} by the oyster, a shell will become radioactive by an exchange of atoms of the shell for those in the surrounding fluid. As a result of this process of exchange a shell carefully cleaned of all tissue will take up increasing amounts of Ca^{45} as it remains in radioactive sea water. The extent of exchange in the shell of the living oyster cannot be estimated precisely without knowing the calcium concentration and specific activity of the fluid in contact with the inner shell surface; but on the assumption that these values are not very different from sea water (Hayes and Pelluet, 1947), an approximation can be made from the radioactivity of cleaned shells placed in the radioactive sea water with the experimental oysters. For the posterior region the exchange so measured was about 1% of the mean and 0.5% of the maximum amount deposited by the living oyster during 24 hours.[6] It is apparent that exchange offers no serious obstacle to the measurement of shell growth.

The non-uniform distribution of newly deposited calcium over the inner shell surface indicated differences in deposition rates which must eventually be correlated with cell structure and mechanisms in localized regions of the mantle. The low rate of deposition in the thin posterior shell margin is especially interesting. This finding does not in itself mean a lower growth rate but perhaps a high organic:inorganic ratio of material deposited. Orton (1928), working with English oysters, mentioned that this thin shell margin hardens later in the season; and Loosanoff and Nomejko (1949) found that the hardness and brittleness of the new growth of Long Island Sound oysters is greater as the environmental temperature increases. These findings strongly suggest that the type of material laid down by the mantle shifts with certain environmental changes. In view of this, the pattern of deposition found in this study might be quite different under other conditions. This could be studied by exposing oysters to Ca^{45} under the desired conditions and making an autoradiographic study of the inner shell surface.

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[6] Exchange in shell will be considered in detail in a separate publication.

Summary

1. The rate of shell formation in the oyster *Crassostrea virginica* Gmelin has been determined by measurements of Ca^{45} deposited by oysters placed in sea water containing Ca^{45}. The experiments were carried out at Beaufort, N. C., during May and June at 21.2 to 25.4° C. Radioactivity measurements were made directly on the inner shell surface.

2. The mean amount of deposition was directly proportional to the time of exposure to Ca^{45} for 8, 16 and 24 hours. Growth increments occurring in four hours were measurable. Individual variation in the rate of deposition was marked.

3. The mean rate of deposition in the posterior region of the shell was approximately twice that in the center and anterior regions. Within a general region local areas may exhibit a still greater difference in rate. From the average radioactivity per unit area of shell surface the total amount of $CaCO_3$ deposited in a given period can be calculated.

4. Shell from which all tissue had been removed became increasingly radioactive in sea water containing Ca^{45} as a result of exchange. The magnitude of exchange was relatively very small as compared with active deposition by the oyster.

5. Advantages and limitations of the isotope method for measurements of growth are discussed.

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